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PRINCIPAL INVESTIGATOR: Joanne Jang

CONTRACTING ORGANIZATION: University of Pennsylvania
Philadelphia, PA 19104

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Joanne Jang

E-Mail: joannej@mail.med.upenn.edu

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14. ABSTRACT:
We have previously demonstrated that MYC or Wnt1 oncogene induction in the murine mammary epithelium results in the formation of mammary tumors. Kras2 mutations in either MYC or Wnt1-induced tumors correlate with oncogene-independent growth while Hras1 mutations do not. Kras2 mutations in MYC and Wnt1-induced tumors also exhibit higher levels of ras and MAPK pathway activation than do tumors that are wild-type for ras or tumors harboring Hras1 mutations. The next phase of this project includes introducing regulatable activated Kras2 and Hras1 alleles into MYC and Wnt1-induced tumors. Although attempts at constructing a mifepristone-inducible system and using retroviral vectors were not successful, future efforts may require creating a different inducible system or adjusting the retroviral infection protocol to manipulate Kras2 or Hras1 independently of MYC or Wnt1.

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c-MYC, transgenic animals, Ras, mammary, carcinogenesis, tumorigenesis

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Introduction

Amplification of the c-*MYC* oncogene occurs in 10-20% of all human breast cancers (*1, 2*). Similarly, overexpression of Wnt oncogene family members and alterations in components of the Wnt signaling pathway are common in human breast cancers (*3, 4*). Our lab has previously established a mouse model of breast cancer that inducibly expresses c-MYC or Wnt1 in the mammary gland (*5, 6*). Induction of either oncogene with doxycycline results in mammary carcinogenesis with an average tumor latency of 20-22 weeks. With the cessation of c-*MYC* expression in the mammary gland, 50% fully regress to a non-palpable state. The remaining 50% resume growth despite the absence of c-MYC expression. In contrast, nearly all Wnt1-induced tumors regress to a non-palpable state upon cessation of Wnt1 expression.

It has been shown that 50% of MYC-induced mammary tumors harbor activating *Nras* and *Kras2* mutations (*5*). Strikingly, activating *Kras2* mutations correlate with progression of MYC-induced mammary tumors to an oncogene-independent state. As MYC is downstream of the Wnt1 signaling pathway, we examined Wnt1-induced mammary tumors for activating *ras* mutations and correlated specific *ras* mutations with oncogene-independent growth following doxycycline withdrawal. Additionally, to assess the contribution of specific *ras* isoforms to tumorigenesis and tumor progression, both MYC and Wnt1-induced tumors, either wild-type for *ras* or harboring different *ras* mutations were biochemically analyzed to determine the activation levels of particular ras effector pathways.

Finally, to study the role of Kras2 in the progression of mammary tumorigenesis, we have been attempting to regulate Kras2 independently of c-MYC and Wnt1, either by building a second inducible transgenic mouse system, or by retrovirally inserting Kras2 into MYC or Wnt1-induced mammary tumor cells. With either method, we will be able to investigate the requirements for mammary tumor induction and maintenance.

Specific Aims:

I. Determine *ras* mutation status in Wnt1-induced tumors and the correlation of *ras* mutations to oncogene-independent growth.
II. Biochemically examine the consequences of *ras* activation *in vivo* in MYC and Wnt1-induced mammary tumors.
III. Develop a mifepristone-inducible transgenic mouse model for breast cancer.
IV. Determine the ability of activated Ras to prevent regression of *MYC*-induced tumors after Myc deinduction.

Statement of Work:

Task 1: Months 1-12: Examine Wnt1-induced mammary tumors for activating *ras* mutations.

This task was completed on schedule. Wnt1-inducible mice were exposed to doxycycline until mammary tumors developed. Tumors were harvested and assayed for activating point mutations in *Hras1*, *Kras2*, and *Nras* (Table 1). In contrast to MYC-induced mammary tumors, in which the majority of *ras* mutations are in *Kras2*, 54% (51/95) of Wnt1-induced tumors harbored activating *Hras1* mutations while 3% (3/95) harbored activating *Kras2* mutations (*Kras2* versus *Hras1*, $p<0.0001$; *Nras* versus *Hras1*, $p<0.0001$).

Task 2: Months 6-18: Correlate *ras* mutation status in Wnt1-induced tumors with oncogene-independent tumor growth.

This task was completed on schedule. Wnt1-induced tumors were biopsied, assessed for *ras* mutation status, and deinduced to monitor regression behavior (Table 2). 93% (28/30) of Wnt1-induced tumors wild-type for *Hras1*, *Kras2*, and *Nras* regressed to a non-palpable state following doxycycline withdrawal. Similarly, 94% (31/33) of tumors with activating point mutations in *Hras1* also regressed to a non-palpable state. In contrast, of three Wnt1-induced tumors identified with activating point mutations in *Kras2*, two grew in a Wnt1-independent manner following doxycycline withdrawal (*Hras1* versus *Kras2*, p=0.0014).

Task 3: Months 12-30: Examine the biochemical consequences of Hras1 and Kras2 activation *in vivo* in MYC and Wnt1-induced tumors.

This task was complete on schedule. Biopsied MYC and Wnt1-induced tumors were examined for levels of ras-GTP, phosphorylated Erk1/2, and phosphorylated Mek1/2. Compared to MYC-induced mammary tumors without detectable *ras* mutations, MYC-induced tumors harboring activating *Kras2* mutations exhibited significantly higher levels of ras-GTP as well as higher levels of phospho-Erk1/2 and phospho-Mek1/2 (Figure 1A). Notably, Wnt1 tumors with activating *Hras1* mutations had significantly, but only modestly, elevated levels of ras-GTP compared to Wnt1-induced tumors without *ras* mutations (Figure 1B). Moreover, no elevations in phospho-Erk1/2 or phospho-Mek1/2 were observed in *Hras1*-mutant Wnt1 tumors. Direct comparison of *Hras1*-mutant Wnt1 tumors to *Kras2*-mutant MYC tumors revealed markedly higher levels of ras-GTP, phospho-Erk1/2, and phospho-Mek1/2 in *Kras2*-mutant MYC tumors (Figure 2A). This comparison also demonstrated that phosphorylation of the MAPK pathway targets p90RSK and Elk1 was elevated in MYC tumors harboring *Kras2* mutations compared to *Hras1*-mutant Wnt1 tumors (Figure 2A). Moreover, consistent with the inference that *Kras2* mutation is a more potent activator of the MAPK pathway than *Hras1* mutation in mammary tumors, Wnt1-induced tumors with activating *Kras2* mutations displayed high levels of ras-GTP that were comparable to those observed in MYC tumors harboring *Kras2* mutations (Figure 2B).

Task 4: Months 1-12: Isolate cDNA clones for *Kras2*.

This task was completed on schedule. RNA was isolated from a MYC-induced mammary tumor with wild-type *Kras2* and used to generate a cDNA library. *Kras2* was cloned by PCR amplification.

Task 5: Months 1-12: Generate cDNA clones with an activating point mutation in *Kras2*.

This task was completed on schedule. Site-directed mutagenesis by PCR amplification of the cDNA clone from Task 1 was successfully used to generate an activating point mutation in *Kras2*, which changes codon 12 from glycine to valine.

Task 6: Months 1-12: Generate expression constructs with GLp65 under the control of the MMTV promoter.

This task was completed on schedule. Constructs for transgenic injection were generated by cloning the activator GLp65 after the MMTV promoter. An internal ribosomal entry sequence (IRES) followed by the coding sequence for a nuclear LacZ (NLS-LacZ) were cloned downstream of GLp65 for use as a reporter of GLp65 expression. Additionally, an MMTV-

GLp65 transgenic mouse (the "MG" mouse line) that has already been characterized was obtained from Dr. Sophie Tsai at Baylor University.

Task 7: Months 6-18: Generate GAL4UAS expression construct for *Kras2*(12V).

This task was completed on schedule. The *Kras2*(12V) point mutant from Task 2 was cloned after a GAL4 upstream activating sequence (GAL4UAS). An IRES followed by the coding sequence for renilla luciferase were cloned downstream of the *Kras2* point mutant to act as a reporter of transgene expression.

Task 8: Months 6-18: Inject expression constructs for GLp65 and *Kras2*(12V) into fertilized mouse oocytes.

This task was completed on schedule. The constructs MMTV-GLp65-IRES-NLS-LacZ and G-*Kras2*(12V) were successfully injected into fertilized oocytes from FVB mice and implanted into pseudo-pregnant mice. Several attempts were made to increase the number of possible founders.

Task 9: Months 9-24: Identify transgenic founder lines for GLp65 and G-*Kras2*(12V).

This task was completed on schedule. Tail DNA from founder mice was screened by PCR to determine the presence of a transgene. Five founders that showed germline transmission were identified from the G-*Kras2*(12V) transgene.

Task 10: Months 12-24: Determine induced and uninduced expression characteristics of transgenic found lines.

This task was completed on schedule. All founders for the G-*Kras2*(12V) transgene were bred to the MMTV-GLp65 transgenic line to test for expression of *Kras2*. Female bitransgenic mice that had both the MMTV-GLp65 transgene as well as the G-*Kras2*(12V) transgene were either given daily intra-peritoneal injections of mifepristone for three weeks or fed chow containing mifepristone for three weeks. Mammary gland tissue was harvested from these bitransgenic mice as well as single transgenic mice induced with mifepristone in the same manner. The tissue was examined by luciferase assay for expression of the *Kras2* transgene. The same testing was completed for the G-*Kras2*(12V, 35S) and G-*Kras2*(12V, 40C) founders.

One G-*Kras2*(12V) founder was found to have expression of Kras2 even without induction with mifepristone, rendering that founder unusable. All other founders were found to have no expression of *Kras2* upon mifepristone induction. Thus the mifepristone-inducible system does not seem to be a viable method of determining whether or not activated *Kras2* is sufficient for MYC or Wnt1-independent tumor growth.

Task 11: Months 12-18: Generate viral constructs with activated Kras2.

This task was completed on schedule. Because transgenic studies were not proceeding as expected, an alternative method to introduce activated *Kras2* into MYC or Wnt1-induced tumors cells was desired. To do this, we planned to retrovirally infect tumor cells in vitro, inject the cells into nude mice on doxycycline, wait for tumors to grow, and then deinduce the mice to assess the ability of the cells to grow independently of doxycycline.

Kras(12V) was cloned into two retroviral expression vectors. One construct is MIGR1-Kras12V, which has *Kras*(12V) under the control of the 5'LTR from MSCV. It also contains GFP as an expression marker for infection under the control of an IRES element. The second

construct is pK1-Kras12V, which also has *Kras*(12V) under the control of the 5'LTR from MSCV. In contrast, this plasmid has a puromycin selection marker under the control of an IRES element so that infected cells can be selected out with puromycin.

Task 12: Months 18-36: Generate virus that expresses activated Kras2 to infect Myc-induced tumor cells in vitro.

This task was completed on schedule. MIGR1-Kras12V and pK1-Kras12V retrovirus was made in 293T cells using the constructs described above as well as additional viral production vectors. MYC and Wnt1-induced tumors were harvested, collagenased, and plated onto plastic petri dishes. It was difficult to make cell lines as tumor cells did not grow well *in vitro*. They did not divide well and eventually progressed to senescence with additional passages. Attempts to infect MYC or Wnt1-induced tumor cells with MIGR1-Kras12V and pK1-Kras12V resulted in very low percentages of infected cells and were largely unsuccessful.

Key Research Accomplishments

- Demonstrated that the majority (54%) of Wnt1-induced tumors harbored activating point mutations in *Hras1* while a smaller percentage (3%) harbored activating point mutations in *Kras2*.
- Demonstrated that as in MYC-induced tumors, *Kras2* mutations in Wnt1-induced tumors correlated with oncogene-independent growth whereas *Hras1* mutations did not.
- Demonstrated that MYC and Wnt1-induced tumors bearing activating point mutations in *Kras2* have higher levels of ras and MAPK pathway activation
- *Kras2* was cloned from *MYC*-induced mammary tumors.
- The point mutant *Kras2*(12V) was generated.
- The construct MMTV-GLp65-IRES-NLS-LacZ was cloned.
- The constructs MMTV-GLp65-IRES-NLS-LacZ and GAL4UAS-*Kras2*(12V) were injected into fertilized oocytes to generate transgenic mice.
- Using the mifepristone-inducible system to express activated Kras2 was found to be unfeasible.
- *Kras2* was cloned into retroviral production vectors MIGR1 and pK1 and retroviral supernatants were produced. Retroviral infection of MYC and Wnt1-induced mammary tumor cells was very inefficient.

Reportable Outcomes

- Poster presentation – 2003 Gordon Conference of Mammary Gland Biology.
- Poster presentation – 2005 Era of Hope DOD Breast Cancer Research Program Meeting.
- Journal article – Jang et al. Isoform-specific Ras Activation and Oncogene Dependence in MYC and Wnt-induced Mammary Tumorigenesis. Mol Cell Biol *accepted for publication* (August 2006).

Conclusions

Our findings demonstrate that MYC-induced mammary tumorigenesis proceeds via a preferred pathway involving *Kras2* mutation, whereas Wnt1-induced tumorigenesis proceeds via

a preferred pathway involving *Hras1* mutation. Notably, while *ras* mutation frequently accompanies both MYC and Wnt1-induced mammary tumorigenesis, the behavior of these two tumor types following oncogene downregulation was found to be markedly different. Our data show that *Kras2* mutation – whether in the context of MYC or Wnt1-induced tumorigenesis – is strongly associated with the acquisition of oncogene-independent tumor growth. In contrast, *Hras1* mutation was not associated with oncogene-independent growth. Our further observation that ras-GTP, phosphorylated Erk1/2 and phosphorylated Mek1/2 levels were all significantly elevated in tumors harboring *Kras2* mutations compared to tumors with *Hras1* mutations suggests a biochemical basis for the differential oncogene independence exhibited by *Kras2*-mutant MYC and *Hras1*-mutant Wnt1 mammary tumors.

Several *in vitro* studies have demonstrated differential activation of ras effector pathways by different ras family members. In a variety of cell types, activated Kras2 stimulates the Raf-1/MAPK pathway more strongly than does activated Nras or Hras1 (37, 40). Conversely, activated Hras1 stimulates the PI3K/Akt pathway more strongly than activated Kras2 (6, 40). Overexpression of specific ras isoforms has also been shown to result in differential cellular behavior, in part through activation of different downstream ras effector pathways (6, 26). Consistent with these *in vitro* experiments, we observed that spontaneous activating *Kras2* mutations in MYC and Wnt1-induced mammary tumors are associated with greater activation of the Raf-1/MAPK pathway than spontaneous activating mutations in *Hras1*. Moreover, tumors harboring spontaneous point mutations in *Kras2* behave differently than tumors bearing *Hras1* mutations as demonstrated by the ability of *Kras2*-mutant MYC and Wnt1-induced mammary tumors to progress to oncogene independence. As such, these models for MYC and Wnt1-induced mammary tumorigenesis provide a novel *in vivo* demonstration of differential activation of ras effector pathways depending on the *ras* isoform mutated.

The introduction of activated Hras1 or Kras2 into MYC or Wnt1-induced mammary tumors would greatly aid our understanding of the role of ras in mammary tumorigenesis and tumor progression. Unfortunately, we have concluded that the mifepristone-inducible system cannot be used successfully to express activated Kras2 in the murine mammary gland. Similarly, retroviral infection of tumor cells resulted in very low infection efficiencies, required *in vitro* manipulation of tumor cells, and did not allow us to temporally control Kras2 expression. In the future, we may be able use a different inducible system to introduce Hras1 or Kras2 into mammary tumors or adjust our retroviral infection protocol to achieve higher rates of infection. These experiments would allow us to examine the ability of Hras1 or Kras2 to confer oncogene-independent growth in MYC and Wnt1-induced mammary tumors.

Taken in concert, our data demonstrate that the nature of a particular initiating oncogenic event plays a major role in determining the specific secondary genetic alterations that are subsequently selected for during the process of tumorigenesis. These secondary events, in turn, play a major role in determining the likelihood that tumors will progress to oncogene independence. Whether such mechanisms of selection are relevant to human tumorigenesis is as yet unclear. However, the fact that preferentially associated sets of mutations have been identified in human cancers, and that a significant number of specific genetic alterations in human breast cancer – including c-*MYC* amplification – have been correlated with aggressive tumor behavior and poor prognosis, suggest that these mechanisms of selection are operative in mammary tumorigenesis in both mice and humans. As such, elucidating the genetic events that contribute to the progression of tumors to oncogene independence may provide insights into the processes by which human breast cancers develop and progress to more aggressive states.

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Supporting Data

Table 1. Frequency of *ras* mutations in tumors from MTB/TWNT and MTB/TOM mice

Transgenic line	*Hras1*	*Kras2*[a]	*Nras*[b]
MTB/TWNT (n=95)	54%	3%	0%
MMTV-Wnt1 (n=10)	20%	0%	0%

[a] *Kras2* vs. *Hras1*, $p<0.0001$
[b] *Nras* vs. *Hras1*, $p<0.0001$

Table 2. *Kras2* mutations are associated with the progression of Wnt1-induced mammary tumors to oncogene-independence.

MTB/TWNT tumors *ras* status	Full Regression	Incomplete Regression
wild-type (n=30)[a]	93%	7%
Hras1 mutant tumors (n=33)[b]	94%	6%
Kras2 mutant tumors (n=3)	33%	67%

[a] wild-type *ras* vs. *Kras2*, $p=0.0024$
[b] *Hras1* vs. *Kras2*, $p=0.0014$

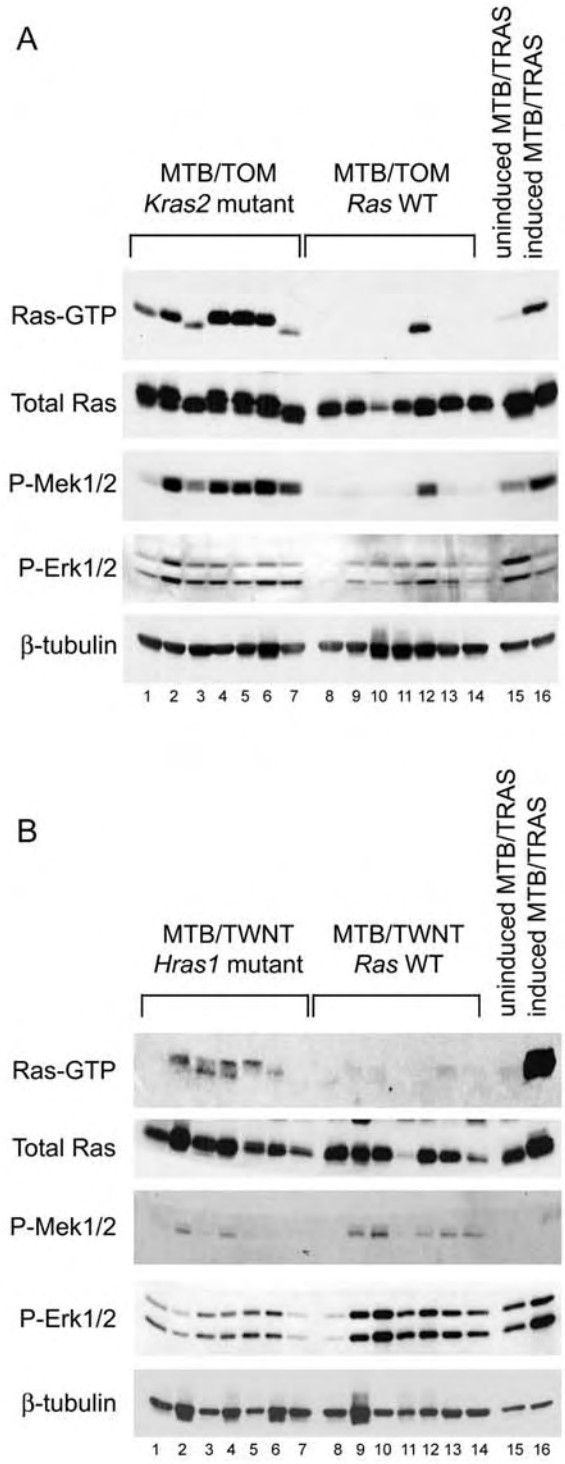

Figure 1. Ras and MAPK pathway activity in Wnt1 and MYC-induced mammary tumors differs according to ras mutation status. A) Immunoblots showing levels of ras-GTP, ras, phospho-Mek1/2, and phospho-Erk1/2 in Kras2 mutant MTB/TOM tumors compared to MTB/TOM tumors without detectable ras mutations. Mammary glands from doxycycline-induced and uninduced MTB/TRAS mice that permit expression of v-Ha-ras in the mammary gland are shown as negative and positive controls. β-tubulin is shown as a loading control. B) Immunoblots showing levels of ras-GTP, ras, phospho-Mek1/2, and phospho-Erk1/2 in Hras1 mutant MTB/TWNT tumors compared to MTB/TWNT tumors without detectable ras mutations. Uninduced and induced mammary glands from MTB/TRAS mice are used as negative and positive controls. β-tubulin is shown as a loading control.

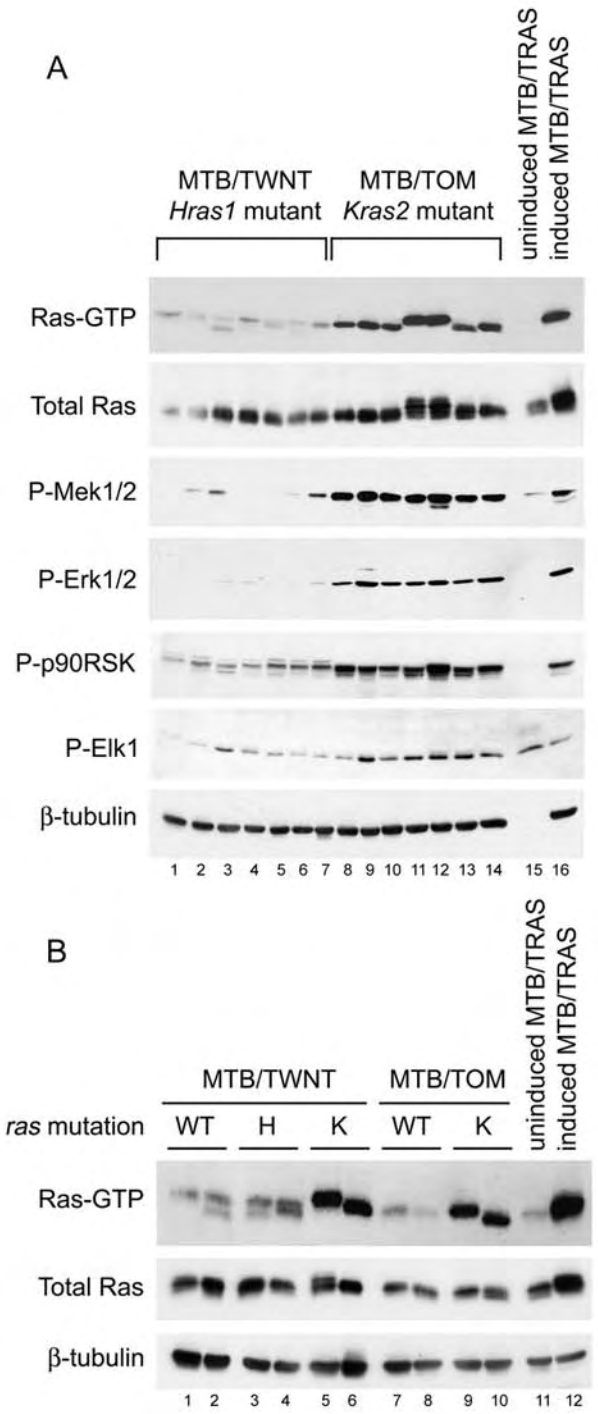

Figure 2. Ras and MAP kinase pathway activity in Wnt1 and MYC-induced mammary tumors bearing mutations in different ras family members. A) Immunoblots showing levels of ras-GTP, ras, phospho-Mek1/2, and phospho-Erk1/2 in Hras1-mutant MTB/TWNT tumors compared to Kras2-mutant MTB/TOM tumors. Uninduced and induced mammary glands from MTB/TRAS mice are used as negative and positive controls. β-tubulin is shown as a loading control. B) Immunoblots showing levels of ras-GTP and ras for MTB/TOM and MTB/TWNT tumors without detectable ras mutations or with Kras2 mutations, and for MTB/TWNT tumors with Hras1 mutations. Uninduced and induced mammary glands from MTB/TRAS mice are used as negative and positive controls. β-tubulin is shown as a loading control.